12.0 MINIMUM PROCEDURAL STANDARDS FOR *IN VITRO* ER TA ASSAYS AND RECOMMENDED SUBSTANCES FOR USE IN VALIDATION STUDIES

12.1 Introduction

A relatively large number of *in vitro* studies have been published on the ability of substances to act as an ER agonist or antagonist. However, the number of substances tested multiple times in the same assays or in multiple assays is extremely limited, precluding a formal analysis of assay performance. In addition, there are no published guidelines for conducting such studies and no formal validation studies have been conducted to assess the performance or reliability of *in vitro* ER TA assays. To assist in the development and characterization of these assays, minimum procedural standards and a list of recommended test substances for use in validation studies are provided. The minimal procedural standards and recommended test substances are based on an evaluation of the multiple *in vitro* ER TA assays considered in this BRD (**Appendix D**, **Sections 6** and **7**). Based on the limited database available, it is difficult to recommend one specific assay for future validation efforts. However, for a more reliable test, it is recommended that an assay with an endogenous or stable ER, using a stable or transiently transfected reporter vector containing the *Luc* gene be used. All test substances must be tested for both agonism and for antagonism (in the presence of the reference estrogen)

12.2 Minimum Procedural Standards

The minimum procedural standards listed below are recommended for standardized protocols developed for various types of *in vitro* ER TA assays. Adequate procedural details are essential to maximize interlaboratory reproducibility and minimize variation that may contribute to erroneous or nonreproducible results.

12.2.1 Transcriptional Activation of the Reference Estrogen

Irrespective of the source of the cell line used, the TA-inducing ability of the reference estrogen (i.e., 17β-estradiol) (**Section 12.2.2**) must be demonstrated. Consistency in the level of the reporter gene product response induced by the reference estrogen is used as a measure of the intralaboratory reproducibility of the assay, and as a criterion for assay acceptance.

12.2.2 Reference Estrogen

Except for one study in which cell proliferation was tested, all the reports to assess ER-induced transcriptional activation used 17β-estradiol as the reference estrogen. Thus, it is recommended that investigators continue to use 17β-estradiol as the reference estrogen in future testing.

12.2.3 Preparation of Test Substances

Test substances must be dissolved in culture medium or in a solvent that is miscible with the medium. For substances not sufficiently water soluble, absolute ethanol or DMSO are proposed as solvents. Preference is given to absolute ethanol since this solvent has been used in most of the studies conducted to date. Other solvents may be used as long as it can be demonstrated that they do not interact, or otherwise interfere, with the test system. A solvent control must be included in each assay.

12.2.4 Concentration Range of Test Substances

To minimize effort and costs in screening/testing, and in recognition that adding excessive amounts of a test substance can perturb the test system through physicochemical mechanisms, most testing schemes include a limit dose (i.e., the highest dose that should be tested in the absence of solubility or toxicity constraints). An agreed upon limit dose for *in vitro* ER TA screening assays has not been established. Historically, the highest dose tested in such assays has ranged from 1 to 100 μM, with most tests conducted using a maximum dose of 1 μM. The EC_{50} values reported for substances tested in various *in vitro* ER TA assays cover eight to nine orders of magnitude (from 20 pM to 8 mM), although the majority of EC_{50} values ranged from 20 pM to 100 nM. Thus, if the *in vitro* ER TA test is required to detect substances with an EC_{50} that is at least 6 orders of magnitude higher than that of 17β-estradiol, then the limit dose (unless precluded by chemical properties such as solubility) should be 100 μM. However, if five orders of magnitude are sufficient for detecting ER agonists, then the limit dose should be 10 μM.

For the *in vitro* screening for ER agonists, it is recommended that the limit dose be 100 μM and that a concentration range from 10 pM to 100 μM, in ten-fold increments, be used in each experiment. However, if it is suspected that the test substance binds weakly to the ER, the dose range should extend up to 10 mM, in ten-fold increments.

For ER antagonism assays, the weakest ER antagonist had a reported IC_{50} value of 0.1 mM. Therefore, the range of substance concentrations tested in such studies should be from 1 nM to 1 mM.

For relatively insoluble substances, the highest dose should be at the limit of solubility and the concentrations tested should then decrease in ten-fold increments. Testing at concentrations that result in precipitation in the test medium should be avoided to minimize false positive results associated with the nonspecific interaction of the precipitate with the receptor (Gray et al., 1997).

12.2.5 Solvent and Positive Controls

Concurrent negative and solvent controls and a reference estrogen must be included in each experiment. The negative control provides assurance that the solvent does not interact with the test system. The solvent should be tested at the highest concentration that is added with the test substance. The reference estrogen in *in vitro* ER TA agonism assays is included to demonstrate the sensitivity of the assay in each experiment for detecting agonist activity and to allow for an assessment of variability in the conduct of the assay across time. In addition, to demonstrate the sensitivity of the *in vitro* ER TA antagonism assay, a substance with demonstrated ER antagonism activity (i.e., a positive control) is needed in each experiment. ICI 182,780 is suggested as the candidate ER antagonist as this substance has historically been shown to be negative as an agonist but positive as an antagonist. For the background antagonist control, ICI 182,780 should be tested in the absence of the reference estrogen. The reference estrogen should be tested alone (positive control) and the reference estrogen with ICI 182,780 as the antagonist control.

12.2.6 Within-Test Replicates

Triplicate values should be obtained for each dose tested, for each control and test substance.

12.2.7 Dose Spacing

Generally, to obtain a response curve to assess ER-induced transcriptional activation, the concentrations of the reference estrogen and the test substances should be spaced by one order of magnitude (i.e., 1 nM, 10 nM, etc.) over the concentration range of interest (1 pM to 100 µM). For antagonists, the concentration range should range from 10 nM to 1 mM. This results in each test of the testing of nine concentrations for agonism and six concentrations of each substance for antagonism. If the range of doses is reduced due to, for example, insolubility of the test substance at the limit dose, then equivalent spacing (e.g., half-log doses) of the nine or six doses over the smaller dose range should be used.

12.2.8 Data Analysis

Different investigators have used various approaches for analyzing data obtained from *in vitro* ER TA assays. For agonist assays, responses are compared to the concurrent vehicle control while for antagonist assays, treatments are compared to the response induced by the reference estrogen alone. Data analysis approaches have varied from a visual inspection of the data only to more formal statistical approaches using either one- or two-way analysis of variance (ANOVA) (with main effects being treatment or replicates and treatment, respectively) using a general linearized model. In some studies, the induced reporter gene response for each replicate has been converted to a fold induction above the concurrent control level, and means and variances of these data used as the basis for analysis. EC_{50} or IC_{50} values have been calculated using various curve fitting programs. One curve fitting approach was based on a logistic dose response model where the asymptotic minimum and maximum response, the dose that is halfway between the minimum and maximum, and the slope of the line tangent to the logistic curve at this mid-point is determined (see Gaido et al., 1997). Asymptotic standard errors of the parameter estimates are employed to perform two-sided "t" tests.

It would be useful, during any future validation study, that various approaches for analyzing *in vitro* ER TA agonist and antagonist data be evaluated and compared in order to develop a standard approach.

12.2.9 Assay Acceptance Criteria

An *in vitro* ER TA assay testing for agonism activity should be accepted only if the response for the reference estrogen occurs within the appropriate confidence limits based on historical data. An *in vitro* ER TA assay testing for antagonism activity should be accepted only if the response for the reference estrogen and the positive antagonism control occur within the appropriate confidence limits based on historical data.

12.2.10 Evaluation and Interpretation of Results

A substance is classified as an ER agonist if the assay-specific response (e.g., luciferase activity) is significantly increased above the concurrent control level, as determined by an appropriate statistical test. A substance is classified as an ER antagonist if the substance induces a significant decrease in the ability of the reference estrogen to induce transcriptional activation, as determined by an appropriate statistical test.

12.2.11 Test Report

At a minimum, the test report must include the following information:

Test substance:

- Name, chemical structure, and CASRN, if known;
- Physical nature (solid or liquid), and purity, if known (every attempt should be made to obtain the purity); and
- Physicochemical properties relevant to the study (e.g., solubility, stability, volatility).

Solvent:

- Justification for choice of solvent if other than medium, absolute ethanol, or DMSO;
- Information to demonstrate that the solvent, if other than medium, absolute ethanol, or DMSO, does not affect the sensitivity of the assay.

Estrogen receptor:

- Type and source of ER (if from a commercial source, the supplier must be identified);
- Isolation procedure or method for making constructs; and
- Nomenclature and components of the expression and reporter constructs.

Reporter plasmid:

- Type of reporter gene;
- Type and structure of response elements;
- Original plasmid used to make construct; and
- Description and methodology used to make plasmid that is transfected.

Cell line:

- Source of cell line and protocol for maintenance of the cell line;
- Growth parameters of the cell line before initiation of the assay; and
- Method used to transfect the reporter construct if it is transiently transfected into the cells.

Test conditions:

- Rationale for the concentration of the reference estrogen used;
- Composition of media and buffers used;
- Concentration range of test substance with justification;
- Volume of vehicle used to dissolve test substance and volume of test substance added;
- Incubation time and temperature;
- Type and composition of metabolic activation system, if added;
- Concentration range of positive and solvent controls;
- Method used to lyse cells after incubation;
- Method used to measure transcriptional activation;
- Methods used to determine fold induction, EC_{50} value for agonism studies, or IC_{50} value for antagonism studies; and
- Statistical methods used.

Results:

- Extent of precipitation of test substance;
- Reporter response for each replicate at each dose for all test substances, including confidence levels or other measure of intradose repeatability;

- Calculated EC_{50} value for agonism studies or IC_{50} value for antagonism studies, and confidence limits, for the reference estrogen (agonism studies), positive control (antagonism studies), and test substance; and
- Fold increase above control for each concentration.

Discussion of the results:

- Historical fold increases in activity and EC_{50} values for reference estrogen (agonism), including ranges, means, and standard deviations; and
- Reproducibility of IC_{50} value of positive control antagonist compared to historical data.

Conclusion:

- Classification of test substance with regard to *in vitro* ER TA agonist or antagonist activity.

12.2.12 Replicate Studies

Generally, replicate studies are not mandated for screening assays. However, in situations where questionable data are obtained (i.e., the fold increase is marginal, the EC_{50} or IC_{50} value is not well defined, the call is equivocal, the test shows excess variability), repeat tests to clarify the results of the primary test would be prudent.

12.3 Standardization of *In Vitro* ER TA Assays for Validation

Appendix B provides six *in vitro* ER TA assay protocols submitted by five investigators. The assay protocols, as titled by the investigators, are:

- Protocol for HepG2 Cells + Receptor + Reporter and/or β-gal Plasmids for Use in Steroid Hormone Receptor Assays, as provided by Dr. Kevin Gaido, CIIT Centers for Health Research, Research Triangle Park, NC, USA.
- Protocol for Chimeric ERα-Mediated Reporter Gene Expression in MCF-7 Cells, as provided by Dr. Timothy Zacharewski, Dept. of Biochemistry, Michigan State University, Lansing, MI, USA.
- Development of New Reporter Gene Assay Systems for Screening Endocrine Disrupters, as provided by Drs. Mitsuru Iida and Teruhisa Kato, Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan.

- Development of Stably Transfected Cell Lines to Screen Endocrine Disrupters, as provided by Drs. Mitsuru Iida and Teruhisa Kato, Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan.
- Technical Perspective on the U.S. EPA Endocrine Disruptor Screening Program: In Vitro EDSTAC Guideline Protocols, as provided by Dr. Grantley Charles, Toxicology and Environmental Research and Consulting, The Dow Chemical Company, Midland, MI, and Dr. William Kelce, Pharmacia Corporation, Kalamazoo, MI, USA.
- Lyticase-based cell lysis protocol of β-Galactosidase assay for 96 well plates, as provided by Dr. Rémy Le Guével of the Université de Rennes, Rennes, France.

Inspection of these protocols provides a perspective on how various *in vitro* ER TA assays are conducted by different investigators. These protocols provide a basis for developing a more general protocol, one that takes into account the recommended minimum procedural standards provided in **Section 12.2**. Prior to developing that protocol, the protocols in **Appendix B** need to be reviewed for completeness and adequacy for their intended purpose.

12.4 List of Recommended Substances for Validation of *In Vitro* ER TA Assays

Tables 12-1 and **12-2** provide recommended lists of substances to be used in the assessment of the reliability and comparative performance of *in vitro* ER TA agonist and antagonist assays, respectively. A number of factors were considered in developing the list for ER agonist studies, including the number of times the substance had been tested in any assay; the median EC_{50} value, when available, of the substance in all assays in which it was tested; the fold increase in response above the control substance; and whether it had been recommended for testing in the ER binding BRD. The latter was considered since it would be informative to assess whether a substance was positive for ER binding but did not elicit a positive transcriptional activation response or vice-versa. For antagonists, the median IC_{50}, if available, and the fold decrease in transcriptional activation compared to the reference estrogen was used. Selection of the substances was based on the availability and concordance of multiple test results among the multiple *in vitro* ER TA assays considered in this BRD (**Appendix E**). When quantitative data was not available for a few substances, consideration was given to qualitative responses (i.e., positive, a weak positive, or negative). Very few substances were tested for their antagonistic properties in these assays.

In a validation study, it is important to include substances that cover the range of possible responses and, therefore this list includes substances in each category. The variability in the numbers of strong, weak, and negative substances in each list reflects the available database.

Table 12-1 List of Substances Recommended for Validation of *In Vitro* ER TA Assays for Agonism

Substance	CASRN	Number of Mammalian Cell Reporter Gene Assays in Which Tested	Median EC_{50} Value (μM) in Mammalian Cell Reporter Gene Assays	Median RBA[a]
17α-Ethinyl estradiol	57-63-6	2	0.000011	
Diethylstilbestrol	56-53-1	8	0.0000189	200
17α-Estradiol	57-91-0	2	0.000046	
17β-Estradiol	50-28-2	46	0.0001	
α-Zearalanol	26538-44-3	2	0.00011	
Estrone	53-16-7	3	0.0032	48
Zearalenone	17924-92-4	8	0.002	44
Methyltestosterone	58-18-4	2	0.0108	
β-Zearalenol	71030-11-0	2	0.015	
Coumestrol	479-13-0	7	0.015	1.9
Estriol	50-27-1	1	0.00071	14.4
4-*tert*-Octylphenol	140-66-9	3	Not available – 0.10 in yeast	0.20
Genistein	446-72-0	11	0.062	0.56
p-Nonylphenol	104-40-5	4	0.0845	
19-Nortestosterone	434-22-0	1	0.212	
Equol	531-95-3	2	0.27	
Daidzein	486-66-8	5	0.29	
Phloretin	60-82-2	4	0.3	
Levonorgestrel	797-63-7	2	0.33	
Bisphenol A	80-05-7	13	0.399	0.056
o,p'-DDT	789-02-6	7	0.66	0.013
Naringenin	480-41-1	5	1.0	0.008
p,p'-DDT	50-29-3	3	Not available – 2.14 in yeast	0.0003

Substance	CASRN	Number of Mammalian Cell Reporter Gene Assays in Which Tested	Median EC_{50} Value (μM) in Mammalian Cell Reporter Gene Assays	Median RBA[a]
Chlordane	57-74-9	1	6.24	
Methoxychlor	72-43-5	12	8.85	0.001
Progesterone	57-83-0	2	negative	0.0003
Atrazine	1912-24-9	3	negative	0.0003
Dicofol	115-32-2	1	negative	
Fluoranthene	206-44-0	0	Not available -- negative in yeast	
Heptachlor	76-44-8	1	negative	
Mirex	2385-85-5	2	negative	

[a] RBA = Median relative binding affinity reported only for substances recommended for use in validating ER binding assays (*Current Status of Test Methods for Detecting Endocrine Disruptors: In Vitro ER Binding*); the median RBA value reported is for positive rat uterine cytosol tests.
Abbreviations: DDT = Dichlorodiphenyltrichloroethane.

Table 12-2 List of Substances Recommended for Validation of *In Vitro* ER TA Assays for Antagonism

Substance	CASRN	Qualitative Response in Mammalian Cell Reporter Gene Assays*
4-Hydroxytamoxifen	68047-06-3	positive (7)
Tamoxifen	10540-29-1	positive (6/7)**
ICI 164,384	98007-99-9	positive (4)
ICI 182,780	129453-61-8	positive (10/11)**
Raloxifene	84449-90-1	positive (5)
Kaempferide	491-54-3	positive (2)
Flavone	525-82-6	positive (2)
Droloxifene	82413-20-5	positive (4)
Hydroxytoremifine	110503-62-3	positive (4)
Dibenz[*a,h*]anthracene	53-70-3	Not available -- positive (2) in yeast
4-Octylphenol	1806-26-4	Not available – positive (1) in cell proliferation
Bendiocarb	22781-23-3	positive (1)
Zearalenone	17924-92-4	positive (2/3)**
Apigenin	520-36-5	positive (2/4)**

Substance	CASRN	Qualitative Response in Mammalian Cell Reporter Gene Assays*
Phloretin	60-82-2	positive (1/3)**
Coumestrol	479-13-0	positive (1/3)**
Formononetin	485-72-3	negative (2)
Bisphenol A	80-05-7	negative (2)
Atrazine	1912-24-9	negative (2)
Fluoranthene	206-44-0	Not available – negative (2) in yeast

Abbreviations: DDT = Dichlorodiphenyltrichloroethane
*Numbers in parentheses refer to the number of different mammalian cell reporter gene assays in which the substance was tested.
** Number of assays in which the substance was positive compared to the number of assays in which it was tested.
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12.5 Summary and Conclusions

Currently, there are no published guidelines for conducting *in vitro* ER TA studies, and no formal validation studies have been conducted to assess the reliability or performance of the currently available assays. To support the further development and characterization of *in vitro* ER TA agonism and antagonism assays, minimum procedural standards for such assays and a recommended list of test substances for use in validation studies are provided. The minimum procedural standards and recommended test substances are based on an evaluation of the *in vitro* ER TA assays considered in this BRD. It is recommended that a mammalian cell assay with an endogenous gene and stably transfected reporter gene, as well as stably transfected plasmid containing luciferase to monitor toxicity be evaluated.

The minimum procedural standards include methods for determining the ability of the reference estrogen to induce transcriptional activation; methods for establishing a stable cell line; the concentration range of the test substance (including the limit dose) to test for agonists and antagonists; the use of negative, solvent, and positive controls; the number of replicates to use; dose spacing; data analysis; assay acceptance criteria; evaluation and interpretation of results; minimal information to include in the test report; and the potential need for replicate studies are described. These minimum procedural standards are provided to ensure that *in vitro* ER TA

studies will be conducted in such a manner as to allow the results to be understandable and comparable among procedures.

Six submitted *in vitro* ER TA assay protocols developed by experts in the field are provided in **Appendix B**. Inspection of these protocols provides a perspective on how various *in vitro* ER TA assays are conducted by different investigators, and for developing a more general protocol, one that takes into account the recommended minimum procedural standards. Prior to developing that protocol, these protocols need to be evaluated for completeness and adequacy for their intended purpose.

A number of factors were considered in developing a list of substances to be used in validation efforts, including the EC_{50} and IC_{50} value of the substance in all of the assays in which it has been tested. The selected substances were sorted according to whether they were positive, weak positive, or negative in at least one *in vitro* ER TA assay.

It is anticipated that this BRD and the guidance it provides will help to stimulate validation efforts for *in vitro* ER TA assays.